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Published in:
Archives of Microbiology

DOI:
[10.1007/BF00414422](https://doi.org/10.1007/BF00414422)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1989

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Veenhuis, M., Sulter, G., Klei, I. V. D., & Harder, W. (1989). Evidence for functional heterogeneity among microbodies in yeasts. *Archives of Microbiology*, 151(2), 105-110. <https://doi.org/10.1007/BF00414422>

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Evidence for functional heterogeneity among microbodies in yeasts

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Abstract. We have studied the biogenesis and enzymic composition of microbodies in different yeasts during adaptation of cells to a new growth environment. After a shift of cells of *Candida boidinii* and *Hansenula polymorpha* from glucose to methanol/methylamine-containing media, newly synthesized alcohol oxidase and amine oxidase are imported in one and the same organelle together with catalase; as a consequence the cells contain one class of morphologically and enzymatically identical microbodies. Similar results were obtained when *Candida utilis* cells were transferred from glucose to ethanol/ethylamine-containing media upon which all cells formed microbodies containing amine oxidase and catalase.

However, when methanol-limited cells of *H. polymorpha* were transferred from media containing ammonium sulphate to those with methylamine as the nitrogen source, newly synthesized amine oxidase was incorporated only in part of the microbodies present in these cells. This uptake was confined to the few smaller organelles generally present at the perimeter of the cells, which were considered not fully developed (immature) as judged by their size. Essentially similar results were obtained when stationary phase cells of *C. boidinii* or *C. utilis* — grown on methanol and ethanol plus ammonium sulphate, respectively — were shifted to media containing (m)ethylamine as the nitrogen source. These results indicate that mature microbodies may exist in yeasts which no longer are involved in the uptake of matrix proteins. Therefore, these yeasts may display heterogeneities in their microbody population.

Key words: Microbodies — Biogenesis — Yeasts — *Hansenula polymorpha* — *Candida utilis* — *Candida boidinii*

In yeasts the proliferation and metabolic function of microbodies (peroxisomes, glyoxysomes and intermediate forms) can readily be manipulated by changes in growth conditions (Veenhuis and Harder 1987). Depending on the final composition of the cultivation medium, organelles may develop which can be involved in the metabolism of the carbon source (van Dijken 1976; Fukui and Tanaka 1979; Veenhuis and Harder 1988), the nitrogen source (Zwart

1983) or both (Zwart et al. 1980; Veenhuis et al. 1985, 1986).

Microbody proliferation has been extensively studied in the yeast *Hansenula polymorpha*. This yeast provides an excellent model system for such studies since it can be grown under conditions where only one small microbody is present per cell (Veenhuis et al. 1979). Transfer experiments have shown that after a shift of such cells to conditions which require one (or more) microbody-borne enzyme(s) for growth, these small organelles serve as a target for newly synthesized microbody matrix enzyme proteins (Veenhuis et al. 1979, 1983; Veenhuis and Harder 1987). As a result of this protein import, the organelles increase in size and subsequently new, small microbodies develop by a process of division. The kinetics of microbody development in methanol-limited *H. polymorpha* following transfer to different growth conditions suggested that organelles, which may be considered mature judged by their size, did not increase in size during subsequent growth of cells and therefore probably no longer incorporated newly synthesized matrix proteins. In fact, these observations suggested that import was confined to newly formed smaller organelles and therefore, with respect to protein import, a population of heterogeneous microbodies might be present in the cells (Veenhuis et al. 1978, 1981). This was also indicated by the results of recent experiments by van der Klei et al. (unpublished data) who studied the recovery of alcohol oxidase activity following the transfer of cyanide-treated cells of *H. polymorpha* into fresh methanol-containing media.

Detailed information on a possible functional and structural heterogeneity of microbodies in yeast cells is of major importance in relation to current studies on microbody biogenesis, particularly those relating to in vivo or in vitro studies on import and assemblage of matrix proteins. In a first attempt to approach this basic question we have now studied the subcellular localization of two substrate-inducible microbody matrix enzymes namely amine oxidase (Zwart et al. 1980) and alcohol oxidase (van Dijken 1976) in *H. polymorpha* and two *Candida* strains, pregrown under conditions where they contained various numbers of microbodies in different stages of development prior to a shift to new growth conditions. The results, presented in this paper, support the view that under certain growth conditions these yeasts contain a population of heterogeneous microbodies.

Materials and methods

Organisms and growth

The experiments were performed with *Hansenula polymorpha* de Moraes et Maya CBS 4732, *Candida utilis* NCYC 321 and *Candida boidinii* (obtained from Dr. Joel Goodman, Dallas University, USA). The organisms were grown in 2 shake flasks at 37°C (*H. polymorpha*) or 30°C (both *Candida* strains) in the mineral medium of van Dijken et al. (1976). After extensive precultivation as described previously (Veenhuis et al. 1979), cells of *H. polymorpha* or *C. boidinii* from the exponential growth phase on 0.25% (w/v) glucose were transferred into media containing 0.5% (w/v) methanol as the carbon source and 0.25% (w/v) methylamine or ethylamine as the nitrogen source. Similarly, glucose-grown cells of *C. utilis* were transferred into ethanol (0.3% v/v)/(methylamine (0.25% w/v) containing media.

In a second series of experiments, cells of *H. polymorpha* and *C. boidinii* from late exponential or stationary phase batch cultures on methanol (0.5% w/v)/ammonium sulphate (0.25% w/v) medium were transferred into fresh methanol (0.5% w/v)/(methylamine (0.25% w/v) containing media. Similar experiments were performed with *C. utilis*, using ethanol (0.3% v/v) instead of methanol as the carbon source.

H. polymorpha was also grown in methanol-limited chemostat cultures (van Dijken et al. 1976). Steady state cultures growing at a dilution rate (D) of 0.07 h^{-1} were shifted from ammonium sulphate to methylamine as the nitrogen source by changing the composition of the inflowing medium which now contained 0.25% (w/v) methylamine instead of ammonium sulphate as the sole nitrogen source.

In the first hours of growth following the shift, samples were taken every 30 min for biochemical and electron microscopical studies.

Preparation of spheroplasts

Spheroplasts were prepared by treatment of suspensions of whole cells with Zymolyase (Kitamura et al. 1971) according to the procedure of Osumi et al. (1975). In experiments designed to demonstrate amine oxidase activity by metal salt methods, the pretreatment of cells with mercaptoethanol was omitted since this compound inhibits the activity of this enzyme (Zwart et al. 1980).

Enzyme assays

Cell-free extracts were prepared by sonification of whole cells (van Dijken et al. 1976). Alcohol oxidase activity was determined as described by van Dijken et al. (1976), amine oxidase activity as described by Zwart et al. (1980) and catalase as described by Luck (1963).

Protein was determined with the method of Bradford (1976) using bovine serum albumin as a standard.

Cytochemical staining

Cytochemical staining procedures for the detection and localization of catalase, alcohol oxidase and amine oxidase activities were performed as described previously (van Dijken et al. 1975; Veenhuis et al. 1976).

Immunocytochemical staining

For the detection of enzyme proteins by immunocytochemical procedures, intact cells were fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 60 min at 0°C, dehydrated in a graded ethanol series and embedded in Lowicryl K4M (Zagers et al. 1986). Immunogold labeling of alcohol oxidase and amine oxidase protein was performed on ultrathin sections by the method of Slot and Geuze (1984) using specific antisera against these proteins. Gold particles were prepared as described by Frens (1973).

Freeze etching

Cell suspensions were harvested by centrifugation, resuspended in a small volume of fresh mineral medium and aerated for 2 min at their normal growth temperature, spray frozen in liquid propane and subsequently freeze fractured in a Balzers freeze-etch unit according to the methods described by Moor (1964), except that instead of Pt an alloy of Ta/W was used (80% tantalum, 20% tungsten).

Fixation and postfixation techniques

Whole cells were fixed in 1.5% (w/v) KMnO_4 for 20 min at room temperature. Spheroplasts were fixed in 6% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 60 min at 0°C. Postfixation — also after cytochemical staining techniques — was performed in a solution of 1% (w/v) OsO_4 and 2.5% (w/v) $\text{K}_2\text{Cr}_2\text{O}_7$ in 0.1 M sodium cacodylate buffer pH 7.2 for 90 min at room temperature. After dehydration in a graded alcohol series the material was embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300 without further staining.

Results

Growth and enzyme kinetics

Growth of cells and induction of microbody-matrix enzymes (alcohol oxidase and amine oxidase) after transfer of glucose-grown cells of *Hansenula polymorpha* and *Candida utilis* to (m)ethanol/(methylamine-containing media is shown in Fig. 1. As is evident from this figure, alcohol oxidase and amine oxidase activities in cell-free extracts are first detected after a period of 2–4 h after the shift. Similar induction patterns were found in parallel experiments with *C. boidinii* and — in the case of amine oxidase only — when stationary phase cells of *C. utilis* or methanol-limited cells of *H. polymorpha* were shifted from ammonium sulphate to (methylamine as the nitrogen source.

Electron microscopy

Methanol-limited cells of *H. polymorpha* ($D\ 0.07\text{ h}^{-1}$) contain many microbodies; the majority of these organelles is cuboid of shape, except those present in the bud (Fig. 2). A shift of such cells from a medium containing ammonium sulphate to one with (methylamine as the sole nitrogen (N) source was not associated with distinct changes in the overall cell morphology. The majority of microbodies continued to be cuboid of shape and showed a completely crystalline

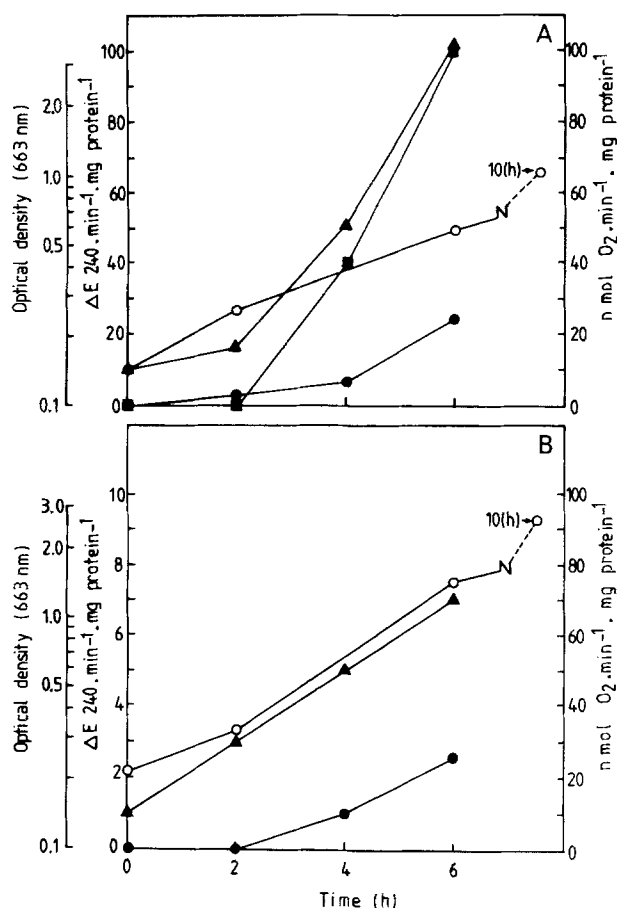


Fig. 1. Growth and enzyme profiles in batch cultures of *Hansenula polymorpha* after a shift of cells from glucose/ammonium sulphate to methanol/methylamine containing media (Fig. 1A) and during growth of *Candida utilis* in batch cultures on ethanol as the carbon source together with ethylamine as the nitrogen source (Fig. 1B). These batch cultures were inoculated with cells from the exponential growth phase on 0.5% glucose ($A = 1.5$). ○—○ growth, expressed as optical density at 663 nm; ▲—▲ catalase; ■—■ alcohol oxidase; ●—● amine oxidase

substructure. However, under these conditions the protein composition of the organelles present in one cell showed distinct differences as became evident from cytochemical studies. After incubation of glutaraldehyde-fixed spheroplasts with CeCl_3 and methylamine in order to localize amine oxidase activity, reaction products were largely confined to the small(er) organelles, which were mainly found at the periphery of the cell (Fig. 3). The larger, cubically shaped organelles generally were completely unstained. On the other hand, the small organelles occurring in developing buds, were all positively stained, indicating that amine oxidase activity was present in each of these organelles 6 h after the shift of cells to the new environment. The presence of amine oxidase protein in these smaller organelles — and its absence in the large cuboid microbodies — was confirmed by immunocytochemical experiments (Fig. 9A). However, when samples were taken from steady state cultures growing on methanol/(m)ethylamine, all microbodies present in the cells again showed both alcohol oxidase and amine oxidase activity, as was shown cytochemically. Similar results were obtained with *C. utilis* and *C. boidinii* grown on alkylated amines as the nitrogen source (Fig. 4 and 9C).

The above results differed from those obtained after a shift of cells grown on glucose to methanol/(m)ethylamine containing media. In *H. polymorpha* the transfer induced a rapid development of the small microbody, originally present (Veenhuis et al. 1979). Cytochemical experiments demonstrated that these organelles now contained, besides catalase, both alcohol oxidase (Fig. 5) and amine oxidase activity (Fig. 6). The labeling patterns, obtained after immunocytochemical experiments suggested that alcohol oxidase protein was mainly present in the crystalloid (Fig. 9B), whereas amine oxidase was mainly in the soluble microbody matrix (Fig. 9D). Essentially similar results were obtained with *C. boidinii* and *C. utilis* in parallel experiments. Also in these organisms all microbodies that developed during the first hours following the shift contained amine oxidase and, in the case of *C. boidinii*, also alcohol oxidase (Fig. 9C). When cells of the three strains were transferred from the late exponential growth phase on (m)ethanol/ammonium sulphate to (m)ethylamine as the N-source, amine oxidase activity was demonstrated in all peroxisomes present in the cells (Fig. 7). However, differences in staining intensity of individual organelles, present in one cell, were evident. Irrespective of the presence of amine oxidase, all microbodies invariably were positively stained after incubations for the detection of catalase activity (Fig. 8).

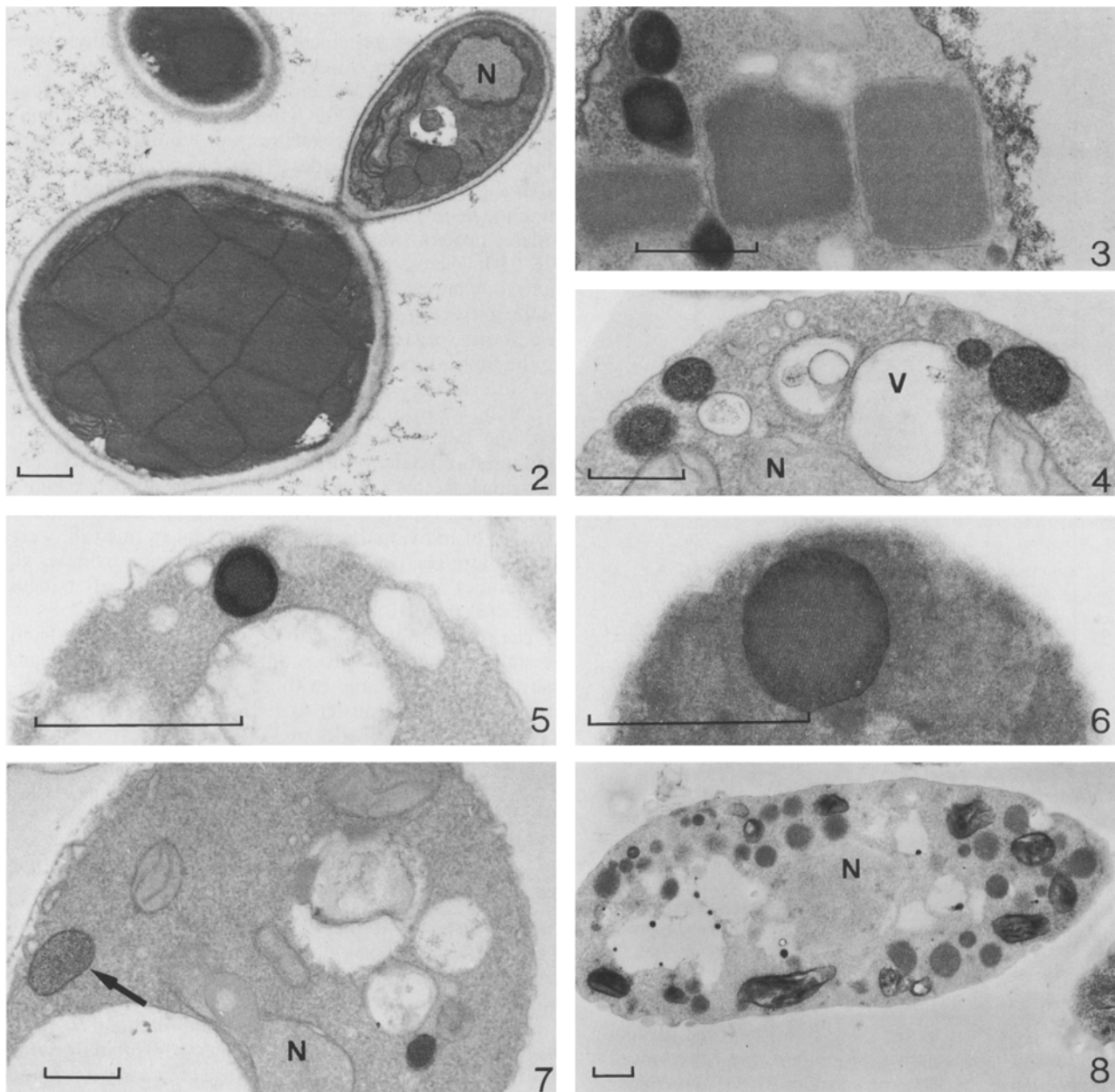
The results, obtained with cells of *H. polymorpha* from the stationary growth phase on methanol/ammonium sulphate, were comparable to those described for methanol-limited cells. After transfer to methylamine as the N-source only part of the organelles present in one cell were stained after incubations for amine oxidase activity. However, in the newly formed cells (which were recognized by their relative thin cell wall and absence of bud scars) again all organelles were positively stained after two doubling times.

Freeze etching

Fracture faces of microbody membranes differ from those of other cell membranes in that they generally do not expose large integral membrane proteins (Veenhuis and Harder 1987). Comparison of fracture faces of membranes of mature organelles (for instance in methanol-limited *H. polymorpha*) with those of smaller organelles (present in the same cells but also the single organelles present in early log-phase cells) did not reveal significant substructural differences. Independent of the growth condition and possibly stage of development of the microbodies, their surrounding membranes showed comparable distribution patterns of small membrane particles (Fig. 10) in all three organisms studied. These particles are generally not visualized after conventional shadowing methods using platinum or platinum alloys (Veenhuis and Harder 1987) but could be resolved in preparations with enhanced resolution obtained after shadowing with tantalum/tungsten alloys.

Discussion

Our present results confirm other observations (Veenhuis et al. 1983) that morphological differences between microbodies present in one yeast cell of a batch- or chemostat culture may reflect differences in the developmental stage of these organelles. In fact the first evidence for heterogeneity among microbodies in one cell was obtained with methanol-limited *H. polymorpha* and concerned morphologically and



Figs. 2 and 3. Section through a methanol-limited cell of *H. polymorpha* ($D = 0.07 \text{ h}^{-1}$) showing the distribution and morphology of microbodies in mothercell and bud. **Fig. 3** shows a detail of such a cell after staining for amine oxidase activity 4 h after a shift from ammonium sulphate to methylamine as the nitrogen source. Only the small, newly formed organelles are intensely stained (CeCl_3 + methylamine). **Fig. 4.** Demonstration of amine oxidase activity in ethanol/ethylamine grown cells of *C. utilis*. All microbodies show comparable staining intensity (CeCl_3 + ethylamine). **Figs. 5, 6.** Details of batch-cultured cells of *H. polymorpha*, showing the staining patterns of alcohol oxidase and amine oxidase activity, 6 h (**Fig. 5**, alcohol oxidase) and 4 h (**Fig. 6**, amine oxidase) after the shift of cells from glucose/ammonium sulphate to methanol/methylamine. **Figs. 7, 8.** In *C. utilis*, transferred in the late exponential growth phase from ethanol/ammonium sulphate to ethanol/ethylamine characteristic differences in staining intensity (arrow) between the large and small microbodies are observed after staining for amine oxidase activity (**Fig. 7**, arrow; CeCl_3 + ethylamine); all microbodies were positively stained for catalase activity (**Fig. 8**; $\text{DAB} + \text{H}_2\text{O}_2$).

Electron micrographs. Abbreviations: *N* nucleus; *V* vacuole; *P* peroxisome. The marker represents $0.5 \mu\text{m}$, unless otherwise stated

enzymatically identical organelles with displayed differences in the activity of one of their major matrix enzymes, namely alcohol oxidase. This heterogeneity was shown to be dependent on cultivation conditions and increased with decreasing growth rates. Especially in cells grown at low growth rates ($D = 0.03 \text{ h}^{-1}$), individual organelles were present in which

alcohol oxidase was fully inactive. In addition, microbodies containing temporarily inactivated alcohol oxidase were encountered in developing buds of methanol-limited *H. polymorpha* (Veenhuis et al. 1978; Veenhuis and Harder 1987). Another example was observed after the transfer of methanol/methylamine-grown cells into glucose/

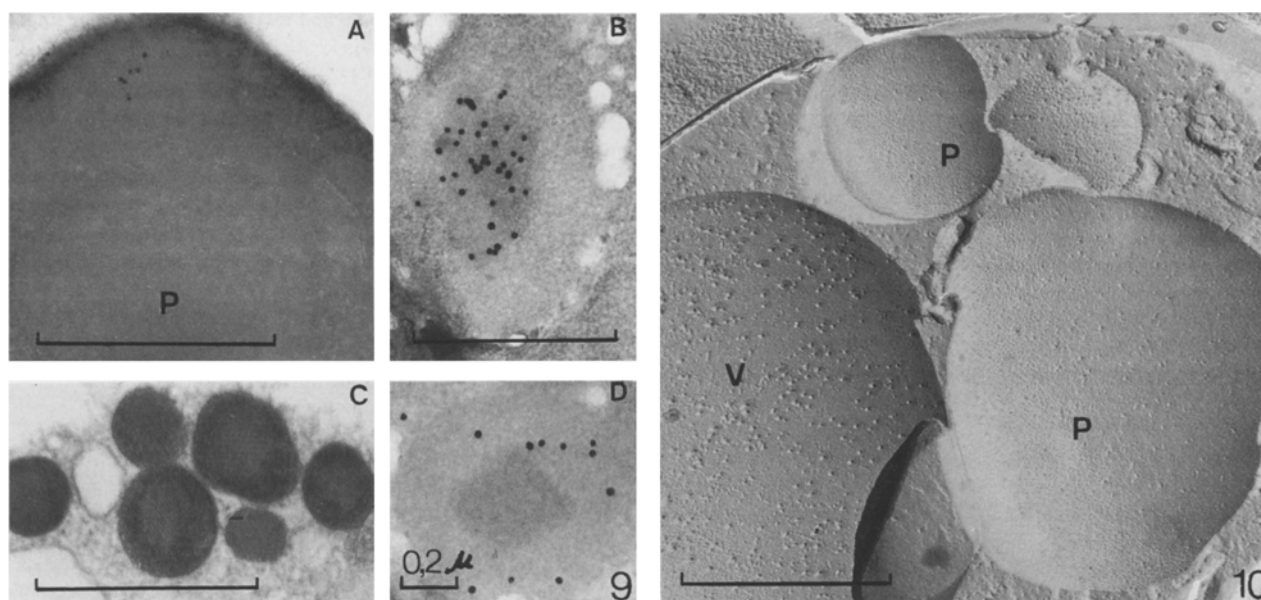


Fig. 9A–D. Details of methanol-limited cells of *H. polymorpha* (Fig. 9A–C) showing the labeling pattern after immunocytochemical staining of amine oxidase protein 2 h after the shift from ammonium sulphate to methylamine as the nitrogen source. Only the small organelle is specifically labeled (Fig. 9A); Fig. 9B and D show the labeling patterns in cells from the exponential growth phase on methanol/methylamine for alcohol oxidase (Fig. 9B) and amine oxidase protein (Fig. 9D). Fig. 9C shows the staining intensity of microbodies present in *C. boidinii* stained for amine oxidase activity, 3 h after the shift of cells from glucose to methanol/methylamine (CeCl_3 + methylamine).

Fig. 10. Freeze etch replica from a methanol-limited cell of *H. polymorpha*, shadowed with Ta/W showing the presence and distribution pattern of small particles on the fracture faces of the membranes surrounding a mature and a relatively small microbody (compare the relatively large particles present on the vacuolar membrane)

methylamine containing media. In the initial hours after the shift new microbodies developed which were characterized by the presence of amine oxidase and catalase (Zwart et al. 1980). Since these newly formed organelles lacked alcohol oxidase activity these cells contained a population of microbodies which was heterogenous with respect to their enzymic contents.

Therefore, the present study is the first report on the occurrence of heterogeneity among yeast microbodies with respect to protein import. Our combined biochemical and (immuno)cytochemical results clearly indicated that microbodies in the three strains studied are — at a certain stage of their development — no longer capable of incorporating newly synthesized matrix proteins. The molecular mechanisms behind this phenomenon are unknown, but may be related to the energy status of the organelles. Nicolay et al. (1987) showed that a pH-gradient exists across the microbody membrane of yeasts, which is most probably generated and maintained by a proton translocating ATPase (Douma et al. 1987). Bellion and Goodman (1987) showed that import and assemblage of alcohol oxidase in microbodies of *C. boidinii* is an energy-dependent process which is prevented by uncouplers. In view of this one may speculate that mature peroxisomes — as for instance present in methanol-limited *H. polymorpha* — are incapable to sufficiently energize their membrane for instance as a result of their relatively large internal volume and high protein concentration thereby rendering these organelles unable of incorporating newly formed proteins. Recently, van der Klei et al. (unpublished data) also observed heterogeneity among microbodies with respect to protein import into cyanide-treated *H. polymorpha*. Knowledge on the nature and composition of the peroxisomal population in eukaryotic cells

is of major importance in relation to current studies on microbody biogenesis. Our present results suggest that especially cells from early exponential batch cultures are excellent model organisms for such studies.

Acknowledgements. Grietje Sulter is supported by the Foundation for Fundamental Biological Research (BION) which is subsidized by the Netherlands Organization for the Advancement of Pure Research (NWO). We wish to thank Klaas Sjollem and Jan Zagers for skilful assistance in preparing the figures and electron micrographs.

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Received June 8, 1988/Accepted July 4, 1988